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Rhizocephalan Parasites of Mud Crabs in South Carolina Estuaries

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Abstract

Rhizocephalan parasites often infect commercially important crustacean species such as the blue crab (*Callinectes sapidus*). In this experiment, the prevalence of rhizocephalan parasites was determined by sampling mud crabs (*Panopeus herbstii*) in three different locations; Huntington Beach State Park, Waites Island, and Murrells Inlet. Crabs were determined to be parasitized by the presence of an externae extruding from their apron. Unparasitized crabs were also collected to serve as a control group. The externa were removed and DNA extractions were performed. Polymerase chain reactions (PCR) were done to prove whether the crabs were parasitized by *Loxothylacus texanus* or *Loxothylacus panopaei*. Results show that all the crabs with externae were infected by a form of *Loxothylacus*. The unparasitized crabs that were sampled for control also tested positive for *Loxothylacus* infection. The restriction digests' results showed that some of the crabs were infected by *L. texanus*, which is supposed to be specific only to the blue crab (*Callinectes sapidus).* Its range is also supposed to be limited to Florida and the Gulf of Mexico, but these crabs were sampled well above that range.

Introduction

The family Rhizocephala includes many species of parasitic barnacles that infect various species of decapods, including crabs of the family Xanthidae. The most prevalent species of parasitic barnacles on the East Coast of the United States are *Loxothylacus* texanus and *Loxothylacus panopaei* (Wardle & Tirpak 1991). *L. texanus* is very host specific, only infecting the blue crab *Callinectes sapidus* (Sherman et al. 2008). It has a

limited range from the northern Gulf of Mexico to Southeastern Florida. *L. panopaei* infects many species of Xanthid crabs, including *Panopeus herbstii*. It has a much larger range than *L. texanus;* from the Gulf of Mexico to the Chesapeake Bay area on the East Coast, and from Southern California to British Columbia on the West Coast (Wardle & Tirpak 1991). Rhizocephalan parasites tend to be found in areas of medium to high levels of salinity. The parasite's larvae die in salinities that are less than 10 ‰ and greater than 60 ‰ (Tindle et al. 2004). Crabs with mature rhizocephalan infections tend to be found in higher salinity areas because these areas have higher partial pressures of oxygen, due to tidal mixing, which aide the parasite in osmoregulation (Robles et al. 2002). Crabs that have immature infections or are uninfected by the parasite tend to be found in all salinity levels that the host normally inhabits (Robles et al. 2002). Xanthid crabs can survive in most salinities but if the crabs are in unnatural conditions for a long time, they come under osmotic stress that can use up energy needed to function (Hulathduwa et al. 2007).

Rhizocephalan parasites are not typical barnacles that attach themselves to a substrate and remain sessile. Rhizocephalans invade their hosts and develop a rootlet system that gradually takes over the host crab's body (Boone et al. 2004). The parasite can change the behavior of the host crab and its sex to use to its advantage. The parasite castrates the crab when the rootlet system grows through the gland that produces the hormones which produce male sexual characteristics (Mouritson and Poulin 2002). Without these hormones, the male crab is essentially female, although the infected individual is sometimes called an intersex (Boone et al. 2004). When a male crab is castrated, it begins to develop secondary female sexual characteristics. The sixth abdominal segment is normally wider in males infected with the parasite because the host

is growing to incorporate the parasite's size and because a wider abdomen is a female secondary sexual characteristic (Knuckey 1995). The general survival strategy of the parasite is to make the male crabs into females in order to give their larvae a better chance at finding another host through the attraction of mates to the crab. The unparasitized male crabs will be attracted to the parasitized crabs because they are behaving like females. This allows the parasite to get close enough to infect the males who are attracted by the faux females. After the crabs have been taken over by the parasite's rootlet system, the parasite begins to develop in the abdominal cavity of the host. An externae develops inside the host until the barnacle larvae are ready to be released into the environment. The externae is a growth of parasitic cells that houses the cypris larvae of the parasite until the conditions are right for their release. Once the larvae are ready, the externae pushes out of a segment in the thorax of the crab and is clearly visible on the apron. The color of the externae holds a clue to the amount of time that the crab has been infected. A white externae means that the parasite larvae are still immature (Wardle & Tirpak 1991). The darker the externae the longer the infection has been going on. The darker brown externae means that the larvae are mature and will soon be released into the environment (Wardle & Tirpak 1991). The development of the externae means that the host crab can no longer grow or molt (Mantelatto et al. 2003). Normally, this makes parasitized crabs much smaller than unparasitized crabs (Alvarez et al. 1995).

The life cycle of a rhizocephalan parasite begins as a cypris larva that is released from the externae of an infected crab into the water. The larva floats in the water column until it comes upon crabs that have just molted. The cypris larvae can only invade the host after they have molted because the shells are soft and vulnerable to attack. Once on

the shell of the crab, the cypris larvae metamorphose into kentrogen larvae which begin to inject themselves into the crab's body via a needle-like cuticle called a vermignon, which is actually a protective cuticle around the parasite's cells (Dillon and Zwerner, 1982). Once inside the host, the kentrogen larvae follow the circulatory system until they reach the abdominal cavity. There, the larvae attach themselves to the host's tissues and begins to grow their rootlet system (Bresciani and Hoeg, 2001). The development of an internae begins after the rootlet system reaches the appropriate size. Once this internae is big enough it pushes out the carapace and becomes exposed to the water. It is now called an externae. The externae is fertilized by male cypris larvae in the water. The larvae inside the externae now begin to cause the color of the externae to change. Once the color turns dark, the cypris larvae are released into the environment (Glenner et al 2000).

The research for my thesis will look at the prevalence of parasitic barnacles in three South Carolina estuaries: North Inlet, Waites Island, and Murrells Inlet. I intend to find out whether the rate of infection by rhizocephalan parasites in this area is higher or lower than rates seen in studies performed in Northeastern Florida. The rates of infection in Florida were anywhere from 1.4 to 17.6% normally, and could get as high as 53% during extreme localized outbreaks (Bortolini and Alvarez, 2008). It would be interesting to see if the same high prevalence would be seen as far north as South Carolina. There have been few studies carried out in South Carolina on the subject of rhizocephalan parasite infection. Since the range of *L. texanus* only extends to Florida, it is more likely that an infection in South Carolina would be caused by *L. panopaei.* Infections by this species have been reported as far north as the Chesapeake Bay. It was hypothesized is that South Carolina mud crab populations had a higher rate of infection by *L. panopaei*

than the rates seen in the study by Bortolini and Alvarez (2008). Evidence for this conclusion comes from the fact that the parasite's range seems to be expanding each year (Kruse and Hare, 2007).

Methods

Thirteen trips to the marsh were organized to collect and measure specimens exhibiting the parasite and a large control population to compare them to. These trips were carried out once in April, three times in May, twice in June, four times in July, and twice in October 2009. There was also one sampling in February, 2010. The reefs were picked out for their large size and accessibility through muddy creek banks. Once a suitable oyster reef was chosen, the crabs found on that reef were collected in plastic containers or buckets. Once the entire reef had been explored for crab activity, the crabs that were found were individually measured for carapace length, sexed, and examined for signs of parasites. The sign of infection by the parasite that was looked for was a visible externae, extending out from the thorax. If the crab is not infected then it was placed back on the reef close to where it was found. If the crab was infected, the color of the externae was noted and the specimen was put in a plastic container and brought back to the lab to be preserved in the freezer. The color of the externae was noted on some outings and not in others so data from that area will not be used. The reefs were surveyed over eleven months in order to determine whether the infection by the parasite is more common in spring, summer, fall, or winter. After the crabs were brought back to the lab, they were defrosted and measured again to collaborate the field notes. Measurements of their

carapace length and the crab's weight were taken, and the crabs were speciated to determine if the parasites were species specific. The location of the reef that the crabs came from was also noted to use for prevalence data later. After the measurements were taken, the externa were removed from the crabs by using a scalpel to separate it from the host crab's apron. The externa were weighed. Each externae was placed separately in a 1.5 ml microcentrifuge tube. To extract the DNA from the externae, 180 μl of Buffer ATL was added to each tube. Then, 20 μl of proteinase K was added to each tube and the tubes were mixed by vortexing them for 10 seconds each. The tubes were placed in a warm water bath set at 56°C for two hours. Every 30 minutes during the water bath, the tubes were removed and vortexed again to mix. Once the two hours was up, the tubes were vortexed again for 15 seconds each. After the mixing, 200 μl of Buffer AL was added to each tube and then the tubes were vortexed again. Then 200 μl of ethanol was added to each tube and they were mixed again by vortexing. From the mixture now in the tubes, 500 μl of it was pipetted into a DNeasy Mini spin column that was placed into a 2 ml collection tube. The samples were centrifuged at 8000 rpm for one minute. This process was repeated until all the samples had been centrifuged. The collection tube for each of the samples was thrown away and the spin column was placed into a new 2 ml collection tube. Five hundred microliters of Buffer AW1 was pipetted into each tube's spin column. The tubes were centrifuged again at 8000 rpm for one minute. The collection tube for each sample was discarded again and a new one added. Five hundred microliters of Buffer AW2 was added to each spin column and then the samples were centrifuged at 13,000 rpm for four minutes. The collection tube was discarded for the last time and the spin columns were placed in 1.5 ml microcentrifuge tubes. One hundred

microliters of Buffer AE were pipetted directly into the membrane in each of the samples. The samples sat at room temperature for one minute and then were centrifuged at 8000 rpm for one minute. The 1.5 ml microcentrifuge tubes used for collection were discarded and a new one placed under all the spin columns. Then, 100 μl of Buffer AE was again added directly to the membrane of the spin column and left to sit at room temperature for one minute. The tubes were centrifuged again at 8000 rpm for one minute. The 1.5 ml microcentrifuge tubes were capped and the spin columns thrown away. Each of the tubes was labeled according to what number the crab was and with the date. The same protocol was followed to extract DNA in February when a control group of unparasitized crabs was collected, except, instead of the externae, a piece of the crab's body tissue was used instead. DNA was extracted from sixty-three parasitized crabs and thirty-one unparasitized crabs.

After the DNA was extracted from both the parasitized and unparasitized crabs, PCR was performed to amplify the DNA. Two master mixes were created for the PCR samples. Master mix one consisted of 18.5 μl of water, 2.5 μl of 10x buffer, 0.875 μl of MgCl$_2$, 0.5 μl of dNTP, 1 μl of the primer H1, 1 μl of the primer 329, and 0.125 μl of Taq polymerase. This recipe was then multiplied by however many samples were being run plus one to make sure there was enough for all the samples. Master mix two consisted of 18.5 μl of water, 2.5 μl of 10x buffer, 0.875 μl of MgCl$_2$, 0.5 μl of dNTP, 1 μl of the primer H1, 1 μl of the primer Loxo3, and 0.125 μl of Taq polymerase. The primers used in this study were H1, 329, and Loxo3. H1 has the sequence 5'-GTG CAT GGC CGT TCT TAG TTG – 3'. Primer 329 is a crustacean DNA primer that includes a DNA sequence that is common to all crustaceans. Its sequence is 5'- TAA TGA TCC TTC

CGC AGG TTC ACC TAC – 3'. Primer Loxo3 is a primer that is specific to a DNA sequence found in the DNA of *L. texanus*. Its sequence is 5'- ACG TTT GAT TGC GCG CGC ACT GTC TGC-3'. The master mixes were put into PCR strip tubes so that each DNA sample had two reactions for itself, one from master mix one and one from master mix two. The strip tubes were prepared with the master mixes accordingly and then 0.5 μl of DNA is added to each tube. The DNA from one crab only goes into two reactions, one from each master mix. Then the next DNA sample continues with its two reactions and so on. After the DNA has been added to the tubes, they are labeled and capped. The tubes are put through the centrifuge to mix the DNA with the master mixes. The strip tubes then go into the thermocycler for 3 hours and 37 minutes. The PCR profile for the thermocycler ran at 95° C for 5 minutes, 30 cycles of 95° C for 40 seconds, 66.8° C for 25 seconds, 72° C for 3 minutes, and then 10 more minutes at 72° C. When the thermocycler finished, the samples were pulled from the machine and placed on ice. 1.5% agarose gels were run to see how the DNA samples came out. While the gel was still hot either 10 μl or 4 μl, respectively, of SYBR gel stain was pipetted into the agarose mixture. The mixture was poured into the gel box and allowed to solidify with combs placed inside to mark the well positions. 100 μl of 1X TBE was poured over the gel to the fill line. Once solid, 8 μl of each sample and 2 μl of loading dye were mixed together and then added to each well. After all samples were loaded into the gel, 5 μl of ladder was added to the first well. The top was placed on the gel box and then the power supply was set to 100 volts. The samples were allowed to run until they reached the end of the gel. The power supply was turned off and the gel was removed from the plate and placed on the light-box. Once the gel was on the light-box, the UV light was turned on with the

shield covering the gel. The results of each gel were recorded by drawing the position of each band on paper and by photographing the gels.

The PCR for these samples was run twice to try and account for anomalies. After the first run of PCR, the PCR products were used to run restriction digests. A master mix was created using 9 µl of nuclease free water, 1 µl of 10x buffer tango, and 1 µl of TaaI. This master mix was multiplied by the number of reactions plus one. Eleven microliters of the master mix was added to each of the 1.5 ml microcentrifuge tubes and then 0.5 µl of the H1/329 PCR product of a crab was added to each tube. These tubes were placed into a heat bath at 37°C for four hours. After the four hours, 0.64 µl of EDTA was added to each tube. These tubes were placed on ice and a gel was run in the same way as the PCR reactions.

Results

In the samples this study took, the average rate of infection is 8%. This comes from the 63 crabs out of 783 that were parasitized. Also, out of the 783 crabs sampled, 763 were *Panopeus herbstii* and 20 were *Petrolisthes armatus*. This means that 97.44% of the crabs caught were *Panopeus herbstii*, while 2.55% were *Petrolisthes armatus*.

From the morphological data gathered when collecting the crabs, we found that the month with the highest prevalence of the parasite is July, followed by October with the second highest prevalence (Figure 1). Out of the total 100 crabs that were found during July, 32 were parasitized. Five crabs out of the 35 found in October were
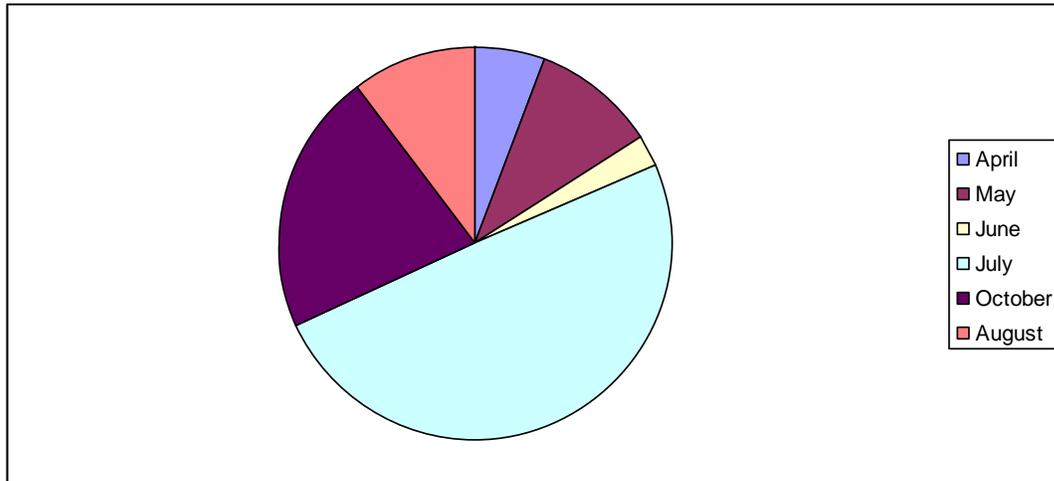
parasitized.

Figure 1: Shows the prevalence of the parasite during each of the months
sampled.

The data from the prevalence of the parasite per each month resembles a normal

distribution when graphed linearly (Figure 2). June is the only anomaly that makes the
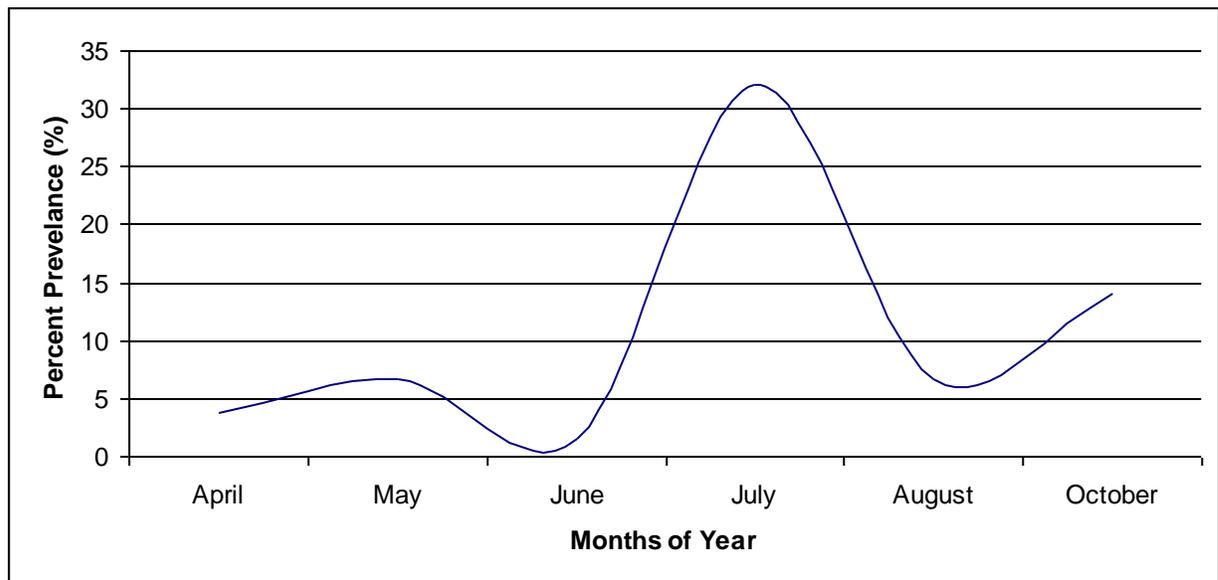
chart not follow normal distribution.

Figure 2: Show the distribution of parasite prevalence per month in an almost
normal distribution.

The morphological data also showed that the location with the highest prevalence of the parasite was the Oyster Landing site near Huntington Beach State Park (Figure 3). This was followed by Murrells Inlet in second place and Waites Island in third. The Oyster Landing site had 55% prevalence, while Murrells Inlet had 17.4% and Waites Island had 16%.
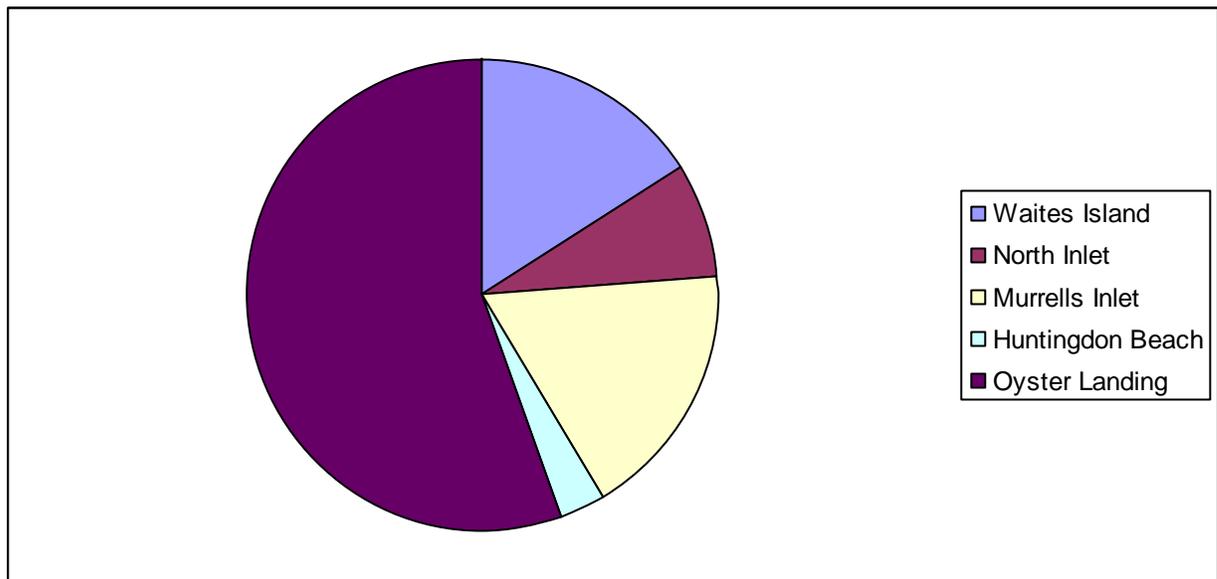

Figure 3: This chart shows the parasite prevalence among sites sampled, with Oyster Landing having the highest prevalence.

Another result obtained from the morphological data is that the average carapace length of the unparasitized crabs is larger than that of the parasitized crabs (Figure 4). The average for the parasitized crabs is 9.133 mm, while the average for the unparasitized crabs is 9.725 mm. The standard deviation of the parasitized crabs was 1.37 mm. The standard deviation of the unparasitized crabs was 5.76 mm. The larger standard deviation for the unparasitized crabs shows that their sizes ranged much larger than those of the parasitized crabs.
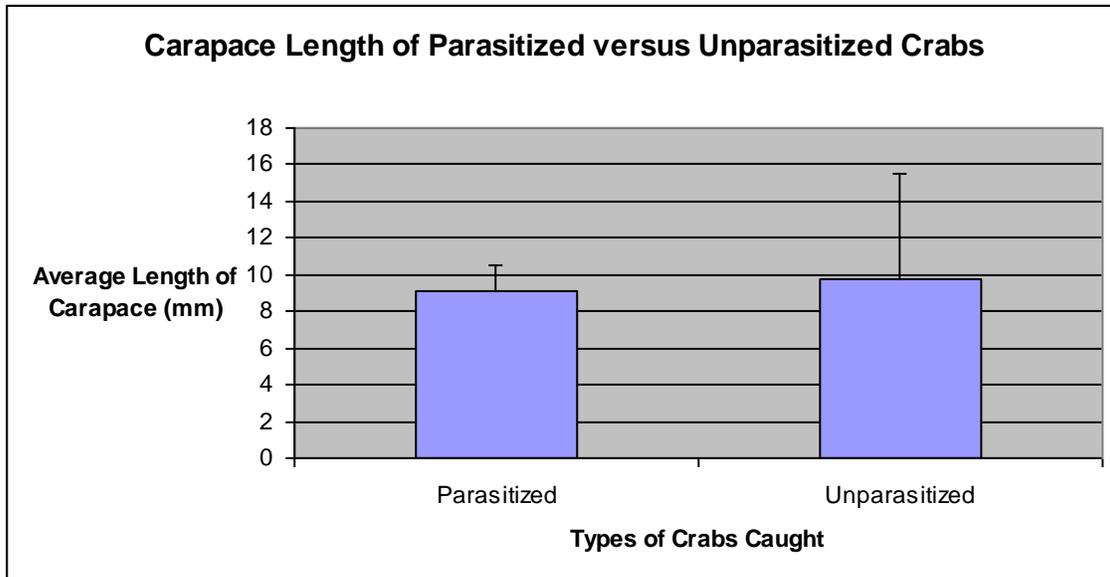
Figure 4: This shows the larger carapace length of the unparasitized crabs versus the parasitized ones.

The results obtained from the gels run from the PCR reactions produced positive bands for all of the samples, even those without DNA included. The bands produced by the H1/329 wells were higher than the bands produced by the H1/Loxo3 wells. Gels were run for all 63 parasitized crabs and 31 unparasitized ones. The results were positive for all wells, making bands show up even in the DNA blank wells. The PCR was run again to try and get a different result but the gel results turned out the same again. Figure 5 is an image of the false positives received in the PCR gels.
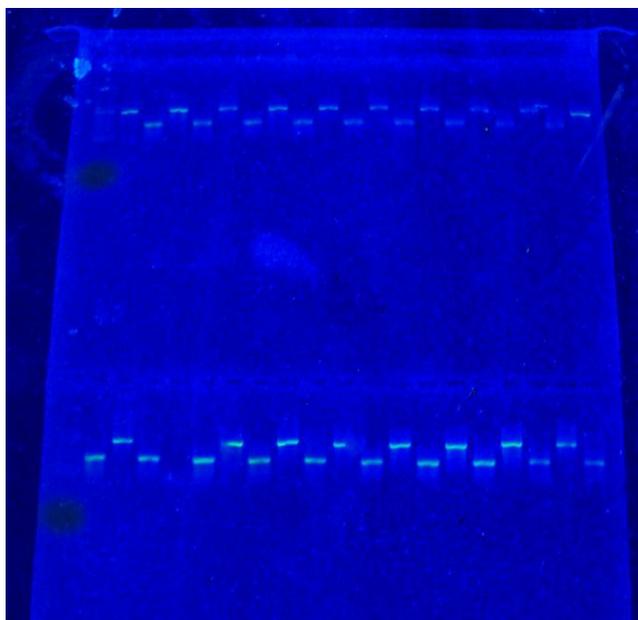
Figure 5: An example of false positives on a PCR gel.

For the few restriction digests that were run, the gel results produced bands of four different sizes in the wells. Three samples were from parasitized crabs while the fourth was taken from the egg mass of a non-parasitized crab. They all produced bands.

Discussion

The prevalence of barnacle parasites in the South Carolina estuaries that this study sampled is 8%. The prevalence of the parasites in Florida waters is 1% (Sherman et al. 2008). South Carolina had a much larger prevalence of the parasite than Florida did. The Sherman et al. (2008) paper had larger sample sizes than in this study, but the relative prevalence was much larger than their numbers. Having a higher prevalence could be because the estuaries in South Carolina are becoming invaded as the range of *L. texanus* changes to include a more northerly area (Kruse & Hare, 2007). It would make sense that the parasite would invade estuaries of similar salinities and tidal fluctuations. Our results also showed that over 97% of the crabs sampled, whether parasitized or not, were *P.*

herbstii. The other three percent was taken up by *P. armatus*, or the green porcelain crab. This species is invasive and originally thought to be from either Brazil or the Gulf of Mexico regions (Masterson, 2007). A 3% rate overall in South Carolina estuaries is a high rate for a species that is invasive.

Our results found that the most prevalent month for parasites was July, followed by October. July had a 32% prevalence which fully supports our hypothesis that more of the parasitized crabs would be found in the summer months, because the water is warmer and therefore allows for the parasite to spread farther because its larvae won't die in cold water (Robles et al. 2002). October also had a high prevalence rate of almost 14%. Because October falls at the end of the summer, when the water begins cooling and conditions begin to change, it makes sense for it to have a lower prevalence than July but still larger than cold months like April and December. Figure 1 shows the distribution of parasite prevalence by month. The data appears to try to follow a normal distribution. There is an outlier in the data, June, which is lower than it should be. This could be because we didn't sample in June as much as we did in it May or July. There was prevalence but it was lower than it should have been because no one was there to document the crabs. The graph also has no data for September because there was no sampling done that month. It would be interesting to see if the values for September were lower than August but higher than October. If we were to follow a normal distribution, the prevalence of September should be in between those values. Figure 2 seems to show a cyclic pattern, which would make sense for the life cycle of a parasite.

Figure 3 shows the prevalence of the parasite at each sampling location. Oyster Landing has the highest prevalence with almost 56%. This could be because this site was

the site sampled at most often. This site was easier to access than the others because it was public access and didn't require payment or a key card to get in the gate. Murrells Inlet and Waites Island were the next most prevalent sites. These two sites were visited an equal number of times and had similar sample sizes each time, which may explain the similarity in prevalence.

Figure 4 shows that unparasitized crabs often grow larger than the parasitized ones. The results of the averages of all the crabs that were sampled seem to support the idea that once parasitized, the host crabs do not get any bigger (Mantelatto et al. 2003), while the unparasitized crabs can keep growing, making their average larger by .6 mm. This is caused by the parasite taking over the crab's body with its rootlet system, which destroys the hormonal systems that tell parts of the crab's body to grow (Boone et al. 2004). None of the parasitized crabs got any bigger than 12 mm, while some of the unparasitized crabs were as large as 23 mm. The standard deviation of the unparasitized crabs (5.76 mm) was much higher than the standard deviation of the parasitized crabs (1.37 mm). This shows that the unparasitized crabs had a much bigger variation in size than those crabs infected with the parasite. This finding fits with the consensus that the parasite makes the host crabs stop growing (Mantelatto et al. 2003).

The PCR results could have come from a variety of causes. The gels constantly showed positive results even with no DNA in them, leads to the first hypothesis of contamination. This hypothesis leads to the question of what could be contaminated. When we first realized we were getting false positives, we started over and reordered everything in the PCR kit and the primers. Extra precautions were taken to avoid contamination, which included wearing gloves at all times, using new pipette tips each

time, and not keeping the reagents in the same place. We walked step-by-step through the process to think of what could be contaminated. The water, 10x buffer, MgCl$_2$, dDNTP, and Taq polymerase were all ruled out due to strict adherence to the following of master mix recipes and getting new ingredients each time. This only leaves the primers as the source of the contamination. This lead us to reorder the primers to try again, but the second try yielded the same results, even with two people redoing the PCR and gels separately. This leads us to believe that the cause of the false positives may not be contamination but a primer that is not specific enough. The primers used in this study were H1, 329, and Loxo3. Primer H1 is common crustacean primer. Primer 329 is a crustacean DNA primer that includes a DNA sequence that is common to all crustaceans. Primer Loxo3 is a primer that is specific to a DNA sequence found in the DNA of *L. texanus*. Our results make us think that perhaps, Loxo3 is not only a sequence that will be in parasite DNA, but all crustacean DNA, which is why it would give positive results when it should give negatives. But, this still doesn't explain why the PCR reactions that were run with no DNA, tested positive with bright bands. One cause of bands could be spillover from lanes with DNA to those without, but the bands that would show up in that situation would be much lighter than those that showed up in our gels. This leads us back to the assumption that the primers must be contaminated. It is the only way for samples with no DNA to test positive and produce bands.

Conclusion

My hypothesis that the prevalence of barnacle parasites was higher in South Carolina than in Florida was proven to be right. The prevalence rates much higher for

South Carolina than Florida, at 8% and 1% respectively. The other purpose of my thesis was to try to determine exactly what species of parasite was infecting the crabs in South Carolina. This, unfortunately, was not possible due to contamination of the primers, which made restriction digests, the step which could have given us that information, impossible to perform. However, the morphological data tabulated from the beginning of my research does show a few very interesting things, such as that prevalence is cyclic in nature and is higher in the warmer, summer months. The data also supported the theory that researchers have that says parasitized crabs are smaller than non-parasitized crabs because the parasite restricts their growth.

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